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A novel *SOD1* splice site mutation associated with familial ALS revealed by SOD activity analysis

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More than 145 mutations have been found in the gene *CuZn-Superoxide dismutase* (*SOD1*) in patients with amyotrophic lateral sclerosis (ALS). The vast majority are easily detected nucleotide mutations in the coding region. In a patient from a Swiss ALS family with half-normal erythrocyte SOD1 activity, exon flanking sequence analysis revealed a novel thymine to guanine mutation 7 bp upstream of exon 4 (*c.240-7T>G*). The results of splicing algorithm analyses were ambiguous, but five out of seven analysis tools suggested a potential novel splice site that would add six new base pairs to the mRNA. If translated, this mRNA would insert Ser and Ile between Glu78 and Arg79 in the SOD1 protein. In fibroblasts from the patient, the predicted mutant transcript and the mutant protein were both highly expressed, and despite the location of the insertion into the metal ion-binding loop IV, the SOD1 activity appeared high. In erythrocytes, which lack protein synthesis and are old compared with cultured fibroblasts, both SOD1 protein and enzymic activity was 50% of controls. Thus, the usage of the novel splice site is near 100%, and the mutant SOD1 shows the reduced stability typical of ALS-associated mutant SOD1s. The findings suggests that this novel intronic mutation is causing the disease and highlights the importance of wide exon-flanking sequencing and transcript analysis combined with erythrocyte SOD1 activity analysis in comprehensive search for *SOD1* mutations in ALS. We find that there are potentially more *SOD1* mutations than previously reported.

INTRODUCTION

Five to 10% of patients with amyotrophic lateral sclerosis (ALS) are diagnosed with a family history of the disease, familial ALS [FALS (1)]. In these patients, the disease is genetically heterogeneous, and mutations in nine genes have been associated with ALS. In most pedigrees, the disease is inherited as an autosomal dominant trait with complete or reduced penetrance, but there are also pedigrees with X-linked and recessive inheritance (2). Mutations in the gene encoding the antioxidant enzyme CuZn-superoxide dismutase (SOD1) have, in different populations, been identified in 12–23% of patients with FALS and in 4–7% of all patients diagnosed with ALS (3). SOD1 is ubiquitously expressed (4) and composed of two equal subunits each containing a catalytic Cu ion and a stabilizing Zn ion (5).

SOD1, localized at 21q22.1, spans 12 kb, consists of five exons and encodes a 153-amino acid polypeptide. Since 1993, 145 mutations have been identified in the gene (http://alsod.iop.kcl.ac.uk/) (6). The majority of these are missense mutations but 13 mutations introduce or delete nucleotides resulting in C-terminal truncations (7–10). With the exception of D90A and possibly also of D96N, all other exonic mutations have been associated with ALS as a dominant trait (11,12). Although most mutations result in a protein with a reduced SOD1 activity, a few mutants have been found to possess essentially normal activity (11,13–15). These findings, combined with the discovery that transgenic mice expressing mutant SOD1s develop motor neuron disease while homozygous SOD1 knockout mice do not, have led to the conclusion that mutant SOD1 cause motor neuron degeneration and ALS because of an acquired cytotoxic property, the nature of which is poorly understood.

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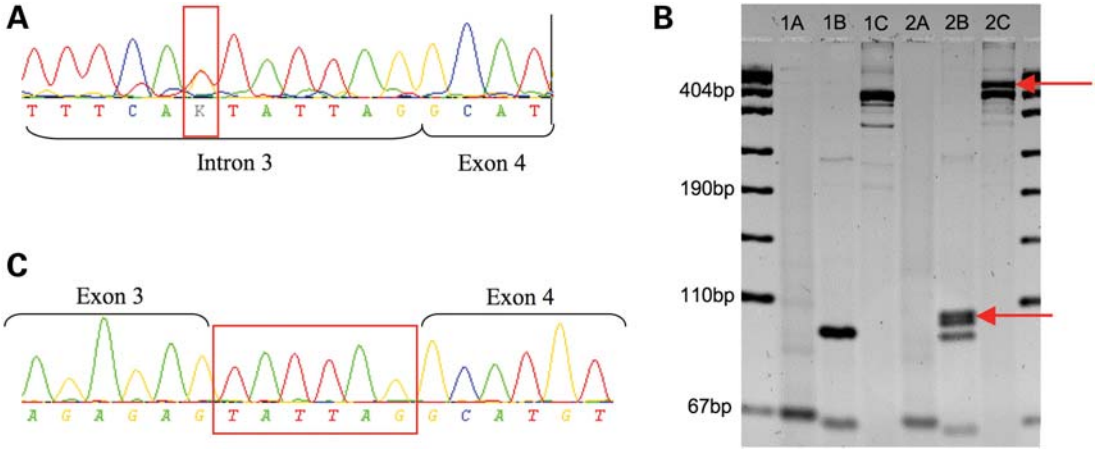

Figure 1. (**A**) Intronic sequence variant found in the FALS patient (heterozygous). The mutation is a single nucleotide exchange, T>G (boxed), in intron 3, 7 bp upstream of exon 4 (*c.240-7T>G*). (**B**) Additional RT–PCR products (arrows) amplified from fibroblasts heterozygous for the intron sequence variant (lanes 2B and C) compared with homozygous wild-type fibroblasts (lanes 1B and C). Primer pair A amplifies a 67 bp product from exon 5 in *SOD1* (positive control). Primer pairs B and C amplify a 96 bp product from exons 3 and 4 and a 395 bp product from exons 2–5, respectively. Sizes of the pUC19 DNA/MspI marker fragments are indicated. (**C**) Sequence of a TOPO-cloned PCR product from the heterozygous cells (lane 2B) showed that one of the amplicons contains the additional bases TATTAG as predicted by the splice site analysis tools SplicePort, SpliceView and HSPL.

A few intronic mutations in the *SOD1* gene have been detected in ALS cases (16–18). Such mutations are troublesome to detect because a comprehensive search necessitates complete intron sequencing. Also, when found, their roles are difficult to interpret. To demonstrate their effect, gene expression studies have to be performed. Intronic *SOD1* mutations can cause splice site aberrations resulting in the deletions or insertions of amino acids as well as truncations. Such perturbations will significantly disrupt the structure and stability of the SOD1 protein and the result will be a major loss of erythrocyte SOD1 activity. Therefore, such analysis will significantly enhance the detection of *SOD1* mutations in ALS. As an example we here report the identification and characterization of a novel intronic mutation in *SOD1* found in a Swiss patient with FALS.

RESULTS

SOD1 gene and transcript

DNA sequence analyses revealed that the FALS patient was heterozygous for a sequence variant in intron 3, a T to G alteration 7 bp upstream of exon 4 (denoted *c.240-7T>G*) (Fig. 1A). The significantly lower (~50% of controls) SOD1 activity in the erythrocytes of the patient indicated that the mutation affected the SOD1 translated from the gene. So, to further investigate the *SOD1* gene, we compared the splice site predictions for the two DNA sequence variants, *SOD1 wild-type* and *SOD1 c.240-7T>G* in seven different splice site prediction analysis tools (Supplementary Material, Table S1). The results of splicing algorithm analyses were ambiguous, but five of the programs predicted that *c.240-7T>G* might introduce a novel acceptor site for exon 4. The mRNA encoded from the new predicted splice variant would have six additional base pairs (UAUUAG) inserted between nucleotides 239 and 240 in the *SOD1* mRNA. To verify the prediction, reverse transcription (RT)–PCR was performed on RNA from fibroblast lines derived from the ALS patient and from the control [spinal and bulbar muscular atrophy (SBMA)]. Agarose gel separation of the amplicons showed that additional products were amplified in RNA from the patient fibroblasts compared with RNA from the control fibroblasts (Fig. 1B). The two mRNAs seemed to be expressed in approximately equal amounts. Sequencing of the larger PCR products (Fig. 1B, lanes 1C and 2C) revealed that the amplicon from the *SOD1* heterozygous cells is a mix of two fragments that differ in sequence with the bases TATTAG. Sequence analyses of the subcloned amplicons (Fig. 1B, lanes 1B and 2B) revealed two different types of fragments. The wild-type *SOD1* sequence and the mutant *SOD1* sequence with the six additional base pairs as expected from the prediction for *SOD1 c.240-7T>G* (Fig. 1C). Since it was only possible to clone two of the three bands visible in lane 2B (Fig. 1B), the third band was concluded to be a heteroduplex between the wild-type and the mutant amplicons.

The intronic sequence variant was not detected in 245 analyzed control individuals. The allele frequency of the variant is thus estimated to be less than 0.002.

SOD1 expression and activity

The mutant mRNA, expressed from the *SOD1 c.240-7T>G* allele, is predicted to encode the mutant protein E78_R79insSI. Western immunoblot analysis of homogenates of fibroblasts derived from the *SOD1* homozygous wild-type control individual (SBMA) and from the heterozygous FALS patient showed a slightly broader band in the heterozygote, suggesting two bands of nearly equal intensities (Fig. 2A). Estimations by Quantity One suggested that the amount of SOD1 in the fibroblast extracts from the FALS patient were 85–90% of that in the control. There was no significant difference between fibroblasts cultured in the presence or absence of the proteasome inhibitor 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone (NLVS).

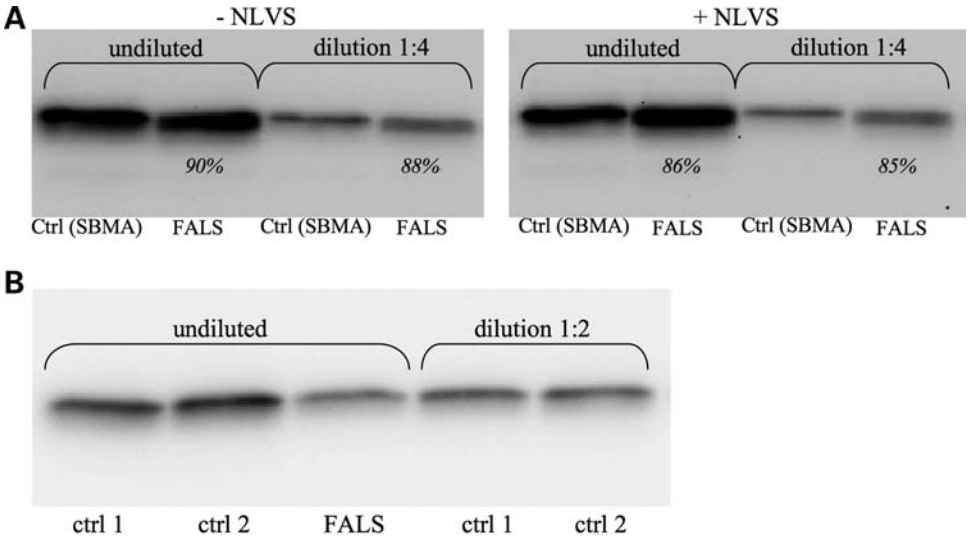

Figure 2. (**A**) Western blot of extracts of fibroblasts, cultured in the presence or absence of NLVS, derived from the control (SBMA) individual and the FALS patient. A slightly broader band is seen in the FALS patient presumably because of the expression of two SOD1 proteins, E78_R79insSI and wild-type, with slightly different migration patterns. The samples were loaded undiluted and diluted 1:4. The relative amounts of SOD1 protein in the FALS samples compared with the corresponding control samples are indicated as percentages. There is no obvious difference between the fibroblasts cultured in the presence or absence of NLVS. (**B**) Western blot of a hemolysate from the patient and the two healthy controls. The amount of SOD1 protein in the undiluted FALS sample is approximately half of that of the undiluted controls (57% estimated by Quantity One) and similar to the amount in the diluted (1:2) controls (88% estimated by Quantity One).

Western immunoblot of a hemolysate from the patient and the two healthy controls showed no indication of two bands in the heterozyguous patient, and the amount of the SOD1 protein was about half of the controls (Fig. 2B). The SOD1 activity was measured in the fibroblast homogenates and hemolysate from the ALS patient and controls. In the hemolysate, the activity in the FALS patient was below 50% of the controls (Table 1).

The SOD1 activity was somewhat lower in the fibroblasts from the ALS patient than in the control. There were, however, no obvious differences in SOD1 activity between the fibroblasts cultured in the presence or absence of the proteasome inhibitor NLVS (Table 2).

DISCUSSION

Genetic screening for *SOD1* mutation in FALS has become a routine in clinical practice and is being performed by several commercial genetic laboratories. Mutations in the *SOD1* gene is the most frequently identified cause of ALS but in different populations only 12–23% of patients diagnosed with FALS and 1–7% of patients with apparently sporadic ALS have been found to carry mutations in the coding region of the *SOD1* gene (2,3,11,18). In the present study, sequencing of the coding regions of the five exons revealed no aberrations but the analysis of the SOD1 activity revealed a 50% reduction. Further analysis showed that the patient was heterozygous for a sequence variant in the third intron of *SOD1*, 7 bp upstream of exon 4 (*c.240-7T>G*). The sequence variant and its context were analyzed for the possibility of additional splice sites in seven different web-based programs. The outcomes of these were ambiguous (Supplementary Material, Table S1). Five of the programs suggested a novel splice site six nucleotides upstream of exon 4 but only in one of the programs was this new site predicted to be the strongest. To examine this possibility, we performed RT–PCR on RNA from fibroblasts heterozygous for *c.240-7T>G*. Indeed, we could confirm that an additional mRNA with the six predicted extra nucleotides was transcribed.

Three splice site mutations have been identified in *SOD1* previously; all residing in the fourth intron (16–18). One of these is particularly interesting since it describes a sequence variant, 304 bp upstream exon 5, that introduces a pseudoexon into the *SOD1* transcript and thus causes a frameshift and a truncation of the protein (16). Mutations that are positioned at a significant distance from the exons are easily overlooked but clearly diseases are sometimes caused by mutations in splice sites or regulatory elements at significant distances from the coding regions. Interestingly, there are also human diseases caused by mutations situated in exons as apparently missense or non-sense mutations but exerting their disease-causing effect by changing the splicing of the gene in question (reviewed in 19–21). These complicated gene expression regulatory mechanisms emphasize the importance to search for *SOD1* mutations in the non-coding regions and of studying the expression of the transcript.

Western immunoblot analyses of fibroblast extracts show that the protein predicted to be encoded by this novel transcript indeed is expressed (Fig. 2A). The slightly broader band in the FALS is presumably because E78_R79insSI has a migration pattern slightly faster than the wild-type SOD1. The insertion should distort the geometry of loop 4 which contains five of the ligands to the prosthetic metal ions and C57 which connects to C146 in a stabilizing intrasubunit disulfide bond. Still the amount of mutant SOD1 protein in the fibroblast extracts was close to that seen in the control both in the absence and presence of NLVS (Fig. 2A), and the mutant protein must possess

Table 1. SOD1 activity in erythrocytes from the FALS patient and controls

Erythrocytes from	SOD1 (U/mg Hb)
Healthy control 1	54.0
Healthy control 2	56.3
Control (SBMA)	63.4
ALS patient	25.3

Table 2. SOD1 activity in fibroblasts from the FALS patient and a control subject with SBMA

Fibroblasts from	± NLVS treatment	SOD1 (U/mg protein)
Control (SBMA)	+	126
	−	170
ALS patient	+	104
	−	120

some enzymatic activity (Table 2). Severely destabilized SOD1 mutants are only detectable in cells cultured in the presence of a proteasome inhibitor (22,23). In contrast, in erythrocytes from the patient, only wild-type SOD1 was found and both SOD1 protein and enzymic activity were half of those in controls (Fig. 2B and Table 1). In the peripheral blood, erythrocytes are on average 60 days old and lack protein synthesis but can eliminate misfolded proteins. A reduction in erythrocyte dismutation activity is therefore a good indicator of the presence of a destabilizing intronic or exonic *SOD1* mutation. The present results suggest that the mutant SOD1 is destabilized, as has been found for other ALS-associated mutant SOD1s. Minute amounts of instable mutant SOD1 protein remaining in erythrocytes has previously been shown to correlate with rapid progression of the disease, which was also seen in our patient (22,24).

The analyses of mRNA (Fig. 1B), fibroblast extracts (Fig. 2A) and erythrocytes (Fig. 2B) combine to show that the usage of the alternative splice site must be close to complete for the mutant allele, which was only predicted by the program FSPLICE at www.softberry.com. This splice site analysis tools also suggested that the normal splice site would be excluded in the mutant sequence. The high usage is in accordance with a high disease penetrance suggested by the many ALS patients in the family. Unfortunately, it was not possible to obtain samples from relatives of the index patient so it could not be formally shown that the mutated allele is causative, nor could the penetrance be evaluated. The mutation was, however, not found in 245 genotyped Swiss control subjects, nor have our laboratory found it in more than 5000 ALS patients and control subjects from Switzerland and other European countries. We suggest that the here reported mutation is a private mutation in this isolated Swiss family and that it is most likely the cause of ALS in the family.

MATERIALS AND METHODS

FALS patient

The proband was a Swiss woman who at the age of 42 years developed leg-onset muscle wasting with rapid generalization to all four limbs and later bulbar innervated muscles. The patient was diagnosed as having ALS according to standard criteria (25). Despite treatment with non-invasive ventilation, the patient expired 20 months after symptom onset. The patient's mother, an aunt and a second-degree cousin had earlier died from ALS. The family is described in greater detail in a separate publication (Weber *et al*., manuscript in preparation).

Human tissue

With written informed consent and adhering to the Declaration of Helsinki (WMA, 1964) venous blood was drawn from the FALS patient, 245 healthy control subjects (blood donors) from the same part of Switzerland and, as a control, a patient with SBMA that had no identified sequence variation in *SOD1*. A skin biopsy was obtained from the ALS patient and the SBMA control patient.

SOD1 genotyping

Genomic DNA was extracted from the buffy coat cells with DNA extraction kit NUCLEON BACC2 (GE Healthcare, Piscataway, NJ, USA) or FlexiGene DNA Kit (QIAGEN, GmbH, Germany) according to the manufacturer's protocol. All five exons and at least 30 bp of flanking intronic sequences were amplified with AmpliTaqGould Kit (Applied Biosystems, Foster City, USA), sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and read in a 3730 DNA Analyzer (Applied Biosystems). The reactions were analyzed with SeqScape v2.5 or in Sequence Scanner v1.0 (Applied Biosystems).

Bioinformatic analysis

The sequence variant found in intron 3 was analyzed for its possible effect on splicing of the *SOD1* transcript in seven different internet-based analysis tools that make splice site predictions for submitted sequences (Supplementary Material, Table S1). The DNA sequence variants *c.240-7T>G* and its sequence context (exon 4 and 100 bp upstream and downstream) as well as the equivalent wild-type *SOD1* sequence were submitted for analysis.

Human skin fibroblast cell lines

The fibroblast lines were established from skin biopsy specimen using standard procedures. The cells were grown to confluence, then passed and kept frozen. For RNA extraction, fibroblasts were seeded in 8 cm diameter culture discs, grown to confluence and then scraped off from the disc and harvested in an isotonic NaCl solution. For SOD1 protein analyses, the cells were grown in the absence or presence of 10 μM proteasome inhibitor NLVS (Merck, Darmstadt, Germany), starting 24 h before harvest. The cells were washed in an isotonic NaCl solution and harvested in APBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) with complete protease inhibitor cocktail with EDTA (Roche Diagnostics, Indianapolis, IN, USA) and

homogenized by sonication using a Sonifier Cell Disruptor (Branson, Danbury, CT, USA).

RNA extraction and RT–PCR

Total RNA was extracted from skin fibroblasts using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's description. RT–PCR was performed using 1 μg total RNA and Titan One Tube RT–PCR System (Roche Diagnostics) according to the manufacturer's description. Primer pairs used were:

(A) 5′-CAGGTCCATGAAAAAGCAGA-3′ and 5′-CGTTTC CTGTCTTTGTACTTTC-3′
(B) 5′-AGGCTGTACCAGTGCAGGTC-3′ and 5′-ACATTG CCCAAGTCTCCAAC-3′
(C) 5′-AGGGCATCATCAATTTCGAG-3′ and 5′-ACAAGC CAAACGACTTCCAG-3′

Primer pairs A, B and C amplify a 67 bp product from exon 5 in *SOD1* (positive control), a 96 bp product from exons 3 and 4 and a 395 bp product from exons 1–5, respectively. The RT–PCR products were separated on a 4% MetaPhor-agarose gel (Lonza, Rockland, ME, USA), stained with Ethidium bromide and scanned using a Typhoon 9400 variable mode imager (GE Healthcare). pUC19 DNA/*Msp*I(*Hpa*II) marker was used as a length marker to estimate the size of the amplicons. The larger amplicons (primer pair C) were sequenced. The smaller amplicons (primer pair B) were cut from the gel, purified with Freeze 'N Squeeze Spin Columns (Bio-Rad Laboratories, Hercules, CA, USA) and subcloned into pCR®II-TOPO-vectors using TOPO TA Cloning-kit (Invitrogen) and top10 competent cells (Invitrogen). The cloned fragments were then sequenced.

Sod1 activity and western immunoblot analysis

The SOD1 activity was analyzed as described previously (26). The amount of protein in the fibroblast homogenates was determined using the Bio-Rad Protein Assay and the amount of hemoglobin (Hb) in the hemolysate was determined with a standard cyanomethemoglobin assay (BR Bioreagens, Ellös, Sweden). The western immunoblots were performed as described previously (22). The volumes loaded were adjusted so that the amount of protein or Hb was equal in all samples. The primary antibody (polyclonal rabbit) used was raised against a peptide corresponding to the amino acids 57–72 in the human SOD1 sequence. The chemiluminescence of the blots was recorded in a ChemiDoc apparatus and the amount of SOD1 protein was analyzed by Quantity One Software (Bio-Rad Laboratories).

Nomenclature

The novel DNA sequence variant was named according to the recommendations for nomenclature for the description of sequence variations proposed by human genome variant society (HGVS, www.hgvs.org) (27). The NCBI Reference Sequence NM_000454.4 was used as a reference for the coding sequence and the A of the first ATG used as residue 1. Since the first Met is excised in mature SOD1, traditional SOD1 nomenclature ignores this amino acid. Therefore, the mutant protein was named E78_R79insSI using the NCBI Reference Sequence NP_000445.1 as a reference and the second encoded amino acid (Ala) as residue 1.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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